PROJECT PLAN PROSPECTUS
NP 106 Aquaculture
Panel Review
June–August 2004

Old CRIS Project Number
6420-32000-012-00D

Research Management Unit
6420-15 Aquatic Animal Health Research Unit

Location
Auburn, Alabama

Title
Molecular Analysis of Virulence Determinants of Select Bacteria in Fish Diseases

ARS Scientists

Victor S. Panangala, Lead Scientist, Molecular Microbiologist…	1.0%
Joel A. Bader, Molecular Biologist……..	.75%
Hung-Yueh Yeh, Molecular Virologist/Immunologist……..	.75%
Thomas Welker, Molecular Physiologist……..	.25%

Scientist at Auburn University funded by ARS

Cova Arias, Molecular Biologist……	.50%

Total Scientific Staff Years
3.25

Planned Duration
60 months

Signatures

/s/ Philip H. Klesius	02/03/04
Research Leader	Date approved
N/A	
Laboratory, Center, or Institute Director	Date approved
/s/ Deborah L. Brennan	02/05/04
Associate Area Director	Date approved
/s/ Lewis Smith	2/06/04
National Program Team Leader	Date approved

Key Words – Fish, Molecular genetics, Microbial adhesins, Virulence, Protease, Antigenic variation, Transcriptome, Pathogenesis, Aquaculture.

Objectives

Objective 1: Identify, clone and characterize adhesins of *Edwardsiella ictaluri* that mediate attachment to the target epithelial cells of catfish.

Objective 2: Molecular genetic and functional analysis of extracellular proteases of *Flavobacterium columnare*.

Objective 3: Molecular genetic analysis of antigenic variation in *Streptococcus iniae* and *S. agalactiae*.

Objective 4: Identify genes that are distinctively expressed *in vivo* by *Edwardsiella ictaluri* when infecting channel catfish.

Objective 5: Examine genes expressed by *Flavobacterium columnare* under *in vivo* and *in vitro* culture conditions.

Need for Research

• *Description of Problem to be Solved*- Fish farmers operate on narrow profit margins and even the modest gains are frequently offset by disease, lowered production, poor carcass quality, and high rates of morbidity and mortality. Among these factors, infectious diseases take precedence over all other contributing causes for production losses. While many of the major aquatic animal diseases have been identified and characterized to some degree, continued loss resulting from microbial infections overshadows the need for innovative molecular biological approaches to study more intricate mechanisms of pathogen/host interactions at the molecular level. Microbial virulence factors (adhesins, toxins), the intricate mechanisms through which the organism outwits (through antigenic variation, production of proteases) the host innate and acquired protective immune systems, invasive traits and pathogenesis remains far from being thoroughly elucidated for most of the major microbial pathogens of fish. *Edwardsiella ictaluri, E. tarda, Flavobacterium columnare*, *Streptococcus iniae, and S. agalactiae* are few among the most important bacterial fish pathogens that continue to pose a severe economic impact on the aquaculture industry in the U.S.A and abroad. With the exception of *E. tarda* and *S. agalactiae* which possess a broad host range, the remaining organisms are primarily fish pathogens.

The objectives presented in this proposal are targeted towards conducting in depth studies on the salient microbial factors vital for production of disease in the host. These include: (1) molecules that promote colonization of the host, (2) epitopes that evade or negate the host immune system and (3) factors that produce tissue damage. Previous studies within the scope of the objectives in this submission fall short or have been dealt from a different perspective. A thorough understanding of the pathogen/host interactions is imperative for the development of efficient and viable strategies for early intervention and protection of fish from devastating diseases. Our studies will result in acquiring vital information on the molecular basis for microbial pathogenesis; consequently, enabling researchers to develop rapid diagnostic test systems and novel, efficacious vaccines. Within the scope of the research that we plan to conduct at the molecular level, there is presently only sparse, incomplete, or a lack of information. Thus the need for this research is timely and could not be overemphasized.

• *Relevance to ARS National Program Action Plan*- This project falls within the Program Component 2 (Integrated Aquatic Animal Health Management) of NP 106-Aquaculture. More specifically addresses goals spelled out in subsections 2.d (Mechanisms of disease) and 2.f (Microbial genomics).

• *Potential Benefits*- Understanding precise mechanisms of pathogen/host interaction is pivotal for development of efficacious disease preventative measures. Overall, our studies are geared towards obtaining important new information on functional genomics and to expand the available genetic sequence information base on hitherto unknown or inadequately understood virulence traits of important fish pathogens. Our studies will directly contribute to accurate gene annotation in whole genome sequencing research that has been completed with *S. agalactiae* and those that are underway with *E. ictaluri*. The long term benefits of our research will impact on the development of rapid, timely and efficient detection of potentially harmful pathogens and thereby negating widespread dissemination of disease. Studies will also augment the development of efficacious vaccines and prevent or minimize the losses due to bacterial infection in aquatic animals.

• *Anticipated Products*- Genomic sequence information on important virulence genes of major fish pathogens will be generated. Functional genomics of pathogenesis and virulence will be realized. Putative genes and gene products that will spawn the development of novel diagnostic tests, production of genetics-based vaccines and potential competitive inhibition of pathogens via intervention by receptor analogs will be explored to eliminate the impact of virulent pathogens in aquaculture.

• *Customers*- Benefits of this research will be realized by fish farmers, other scientists working to improve aquatic animal health, and the aquaculture industry as a whole. Possible technology transfer opportunities that would emerge from this research will enable mass-scale commercial production of therapeutic or prophylactic products that will ultimately benefit the fish farmers, consumers and the industry.

Scientific Background

The researchers on this project at the Aquatic animal Health Research Unit are a cohesive group possessing broad expertise in microbiology, molecular biology, immunology and aquaculture techniques. The equally competent collaborating scientists have been interacting cooperatively on several ongoing research projects. Portions of this project will be closely coordinated with the “Aquatic Animal Diagnostics, Pathogenesis and Applied Epidemiology” (CRIS # 6420-32000-012-00D) and the “Vaccinology and Immunity of Aquatic Animals” (CRIS# 6420-32000-012-00D) submitted by the AAHRU. A perusal of the CRIS, Medline and Aquatic Science & Fisheries Abstracts (ASFA) databases revealed no perceived duplication in approach or methodology pertaining to the specific organisms in our planned research. The competence and experience of the scientific team and the excellent facilities available at the AAHRU will reflect that we are well positioned to undertake this research.

Objective 1

Hypothesis – Attachment of *E. ictaluri* to host epithelial cells mediated by adhesins is a prerequisite for pathogenesis and disease in catfish.

• *Experimental design*- Identify adhesin(s) pilin and/or membrane proteins that mediate attachment of *E. ictaluri* to the host mucosal epithelial surface by attachment assays using cultured cells derived from target organs of channel catfish and established cell lines. Conduct competitive inhibition assays using a panel of monoclonal antibodies (MAbs) that we have developed to membrane proteins of *E. ictaluri*. MAbs that inhibit attachment will be used to screen a genomic DNA library of *E. ictaluri* developed in *Escherichia coli* using the λ ZAP II vector. Disruption of the adhesin gene of *E. ictaluri* will be attempted by site-directed, transposon insertion, or PCR mutagenesis and mutants compared with the parents in attachment assays as described above. Probe the cognate receptor on the host cell membrane and use receptor binding analogues to block adherence.

• *Contingencies*- As an alternative to MAb-screening, to detect clones carrying the genes of interest, we would affinity purify the adhesin protein(s), transfer them (Western blotting) onto PVDF membranes and subject the protein to automated Edman sequence analysis. The N-terminal sequence and portions of the internal amino acid sequences will be determined. On the basis of protein sequence data, through a strategy combining oligonuleotide primer construction, PCR, and DNA sequence analysis, the partial sequence of the putative adhesin of *E. ictaluri* will be determined. Probes based on this sequence will be used to screen the genomic DNA library constructed as described above. This strategy has been used successfully in the past.

Objective 2
Hypothesis – Immune response in fish is mitigated by three distinct proteases of *F. columnare.*
• *Experimental design*- The nucleic acid sequence of one of the 3 proteases of *F. columnare* have been identified, cloned and sequenced by us. As a logical extension to our previous studies, we will construct genomic DNA libraries to identify and characterize the other two types of *F. columnare* proteases. A comparative analysis of proteases from pathogenic and non-pathogenic isolates of *F. columnare* will be conducted. Mutant *F. columnare* will be generated using TN10, chemical mutagens and antibiotics and these isolates evaluated for protease production. The available protease gene sequence will be used to detect pathogenic strains of *F. columnare* with the real-time PCR technique. We will evaluate the action of proteases on the host immune response based on techniques used for IgA proteases produced by other Gram negative organisms.

• *Contingencies*- Modify or redesign methodologies to increase the yield and/or purity of the proteases by applying fermentation technology and reverse phase chromatographic procedures.

Objective 3
Hypothesis – Distinct genomic groups of *S. iniae* and *S. agalactiae* show variability in their pathogenicity to fish
• *Experimental design- Streptococcus* isolates available in our laboratory collection and additional *S. iniae* and *S. agalactiae* isolates obtained from the American Type Culture Collection will be used. The 16S rDNA, 16-23S intergenic spacer region (ISR), and the recA gene of *S. iniae* and *S. agalactiae* will be analyzed by amplified fragment length polymorphism (AFLP), pulsed-field gel electrophoresis (PFGE) and DNA-DNA hybridization. Distinct genomic groups will be identified and analyzed for variability in pathogenesis by challenge studies with tilapia. On the basis of pathogenesis in distinct groups, key virulence determinants will be identified using cDNA-AFLP expression profiles. Complete genome sequences of *S. agalactiae* are presently available in the National Center for Biotechnology Information, GenBank. Genome sequence information will be used to construct DNA microarrays containing all expressed sequence tags (ESTs) of *S. agalactiae* for gene expression analysis and identification of genes important for pathogenesis.

• *Contingencies*- Success of genotyping analysis will rely on selection of restriction enzymes to obtain the highest resolution between genomes. Initially, restriction enzymes will be virtually selected with the web-based program (http://www.in-silico.com) using the genome of *S. agalactiae*. Another problem is combining information from several genomic analyses. As a solution, available BioNumerics software will be used to combine genomic data for analysis.

Objective 4
Hypothesis – In vivo expressed virulence genes of *E. ictaluri* play a significant role in pathogenesis in fish.

• *Experimental design*- To identify *in vivo* expressed virulence genes of *E. ictaluri*, two main strategies will be used, i.e., in vivo expression technology (IVET) and signature tagged mutagenesis (STM). IVET is a promoter-trap strategy designed to identify genes whose expression is induced in a specific environment, typically the host. STM uses comparative hybridization to isolate mutants unable to survive in certain environments and can be used to identify genes critical for survival in the host. Both approaches, using standard techniques, will be applied in *E. ictaluri* to better understand its mechanisms of pathogenesis. Finally, we will identify genes encoding for epitopes expressed by *E. ictaluri* only when grown *in vivo.* Serum from *E. ictaluri* infected catfish will be absorbed with *E. ictaluri* cells grown *in vitro*, thus, subtracting the *in vitro*-specific-antibodies. This serum will be used to probe expression genomic libraries of *E. ictaluri* and positive clones will be fully characterized.

• *Contingencies*- The perceived obstacle to the first strategy hinges on the need for a functional reporter system for IVET. Since we lack an auxotrophic mutant at this stage, we will incorporate an antibiotic resistance gene in the cassette. We plan to simplify STM screening by using tagged transposons which will eliminate the need for prescreening.

Objective 5
Hypothesis - (1) Virulence-associated genes are distinctive in pathogenic strains of *F. columnare* compared to that of avirulent strains. (2) Unique genes are expressed in vivo, when *F. columnare* colonize catfish.
• *Experimental design*- Two approaches will be used to identify virulence-related genes of *F. columnare*. First, we will compare whole transcriptomes of two *F. columnare* strains (a virulent and an avirulent mutant) while growing *in vitro*. Both mRNA populations will be compared by cDNA-AFLP analysis. Genes expressed only by the virulent or by the avirulent mutant will be isolated, cloned, and sequenced. The second approach aims to identify genes expressed by *F. columnare* when colonizing channel catfish. To achieve this objective, we will compare cDNA populations expressed by the same isolate when growing *in vitro* and *in vivo*. A genomic library of *F. columnare* will be generated, spotted on to microarray slides and hybridized with cDNA probes representing both populations. Clones representing differentially expressed genes will be identified and sequenced.

• *Contingencies*- High quality mRNA is essential for cDNA construction. Since bacterial mRNA is poorly polyadenylated and use of oligo (T) to generate cDNA is not feasible, we will remove ribosomal RNA from the sample using capture probes against the 16S and 23S rRNAs. Spotting sufficient clones on microarrays to achieve full genome coverage will be problematic. Since a shot-gun library will be used, we estimate ≈ 15,000 clones (assuming a 1 kb insert size) will provide sufficient genome coverage.

Collaborators
Non-ARS- Vicky van Santen, molecular biologist/virologist at the Department of Pathobiology, Auburn University, and Kevin Dybvig, molecular biologist at the Molecular Genomics lab at the University of Alabama at Birmingham will provide their expertise in the construction of genomic libraries and mutation studies. Zhanjiang Liu, Molecular biologist at the Department of Fisheries and Allied Aquaculture, Auburn University will serve as a consultant with the AFLP and microarray experiments. **ARS-** Brian Scheffler, molecular biologist at the USDA/ARS-MSA Genomics Laboratory will provide assistance in gene sequencing and Geoff Waldbieser, molecular biologist, USDA/ARS, will serve as a consultant on overall genomics (both at Stoneville, Mississippi).